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# GEL CHROMATOGRAPHY OF THYROIDAL IODO-COMPOUNDS ON SEPHADEX G-10

## SEPARATION OF IODOPEPTIDES, IODIDE, 3-MONOIODOTYROSINE, 3,5-DIIODOTYROSINE AND IODOTHYRONINES

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## SUMMARY

The analysis of biological samples containing iodo-compounds was developed by combining partition chromatography and molecular sieving. Iodopeptides, iodide, 3-monoiodotyrosine, 3,5-diiodotyrosine and thyroid hormones are separated in a two-step single-column procedure using Sephadex G-10. The results demonstrate a good reproducibility for $K_{av}$ and also for recoveries.

The main advantages of this new technique are the achievement of total analysis on a single column, direct chromatography of the sample without pre-treatment (lipid removal, iodide removal, etc.), and the possibility of chromatography of large volumes.

Applications to thyroid dialyzates and hydrolyzates are discussed.

## INTRODUCTION

Various techniques have been already published for the separation of iodo-compounds of biological interest: iodide, 3-monoiodotyrosine (MIT), 3,5-diiodotyrosine (DIT), triiodothyronine (T3) and thyroxine (T4). However, none of them is suitable for the direct and complete analysis of a biological sample. Some techniques have been published only for radioactive standards[1–4]. When a biological sample is to be analyzed, a pre-treatment is generally needed either for removal of lipids[5] or for concentration[6,7]; also, the removal of iodide is sometimes necessary before chromatography[5,6]. Paper chromatography[8] and thin-layer chromatography[9] frequently show intermediate spots when spraying the chromatogram, which renders the determination of iodoamino acids difficult. The same situation is encountered with gel chromatography on Sephadex G-25, where iodopeptides migrate with and between iodotyrosines and iodide[10].

The purpose of the present work was to develop a new technique that might be suitable for the direct and quantitative analysis of any biological sample. This

technique allows the separation of iodopeptides, iodide, MIT, DIT and thyroid hormones using a single column of Sephadex G-10.

## MATERIALS AND METHODS

### *Reagents*

All reagents were of analytical grade and were purchased from E. Merck (Darmstadt, G.F.R.), except for iodide (Prolabo, Paris, France) and pancreatine (Organo-Technic, Melun, France). Water distilled in an all-glass apparatus was used. The mixtures used as eluents were as follows:

*Eluent 1.* 0.2 *M* ammonium acetate solution, pH 5.8.

*Eluent 2. tert.*-Amyl alcohol saturated with 2 *N* ammonia solution[11].

*Eluent 3.* Aqueous phase of Partridge's solvent (see eluent 4) as obtained by dilution with water (1:5).

*Eluent 4.* Partridge's solvent: *n*-butanol–water–acetic acid (78:17:5).

*Eluent 5.* Eluent 3 supplemented with carrier iodide (10 $\mu$g/ml) and sodium chloride (0.15 *M*).

### *Radioactive standards*

These were obtained from the Commissariat à l'Énergie Atomique (Saclay, France). T4 labelled with $^{131}$I (average specific radioactivity 70 mCi/mg), T3 labelled with $^{125}$I (average specific radioactivity 25 mCi/mg), MIT labelled with $^{125}$I (average specific radioactivity 26 mCi/mg) and DIT labelled with $^{125}$I (average specific radioactivity 10 mCi/mg) were used as radioactive standards for gel chromatography. Iodide labelled with either $^{131}$I or $^{125}$I was obtained carrier-free.

Labelled molecules were stored at 4° immediately after they were received. No serum albumin was added as a stabilizer because its presence may affect the gel chromatography of these compounds and so modify their elution volume. By the first day of utilization, the average extents of deiodination were 6% for MIT and DIT, 2% for T3 and 10% for T4. After longer storage delays, the maximum extents of deiodination were 12% for MIT, 16% for DIT and 7% for T3 after 3 months and 20% for T4 after 1 month. These high values, particularly for DIT and T4, did not affect the measurements of the elution volumes.

A few microlitres of each solution of radioactive standards were diluted in either 0.09 *M* phosphate buffer of pH 7.2 or 0.2 *M* ammonium acetate buffer of pH 5.8 just before chromatography.

### *Animals*

Male rats of the Wistar strain were housed at 23 $\pm$ 1° and were adapted to receive 50 $\mu$g of iodine daily during at least 1 month. When used, they weighed about 250 g. One or two days before sacrifice, they received one intraperitoneal injection of 50–100 $\mu$Ci of carrier-free $^{125}$I diluted in 0.2 ml of 0.9% sodium chloride solution.

### *Dialyzates of in vivo labelled thyroid*

Whole thyroid lobes of rats were dialyzed with 10 ml of 0.01 *N* ammonia solution per lobe at 4° for 24 h[12,13]. The dialyzate was concentrated *in vacuo* at a temperature below 40° after the addition of 1 $\mu$g/ml of thiouracil in order to prevent

deiodination. Usually, the dialyzate was concentrated to about one hundredth of its volume and dissolved in phosphate buffer or ammonium acetate solution before chromatography. The sample contained mainly iodopeptides accompanied by iodide, MIT and DIT.

*Hydrolyzates of in vivo labelled thyroid*

Thyroid lobes of rats were homogenized in a 0.09 *M* phosphate buffer of pH 7.7 in the presence of thiouracil (final concentration $2 \cdot 10^{-3}$ *M*). After the addition of one drop of toluene, the homogenate was hydrolyzed at 37° with pancreatine (0.5%, w/v) at a tissue concentration of 6% (w/v)[14] for 15 h. This time was chosen so as to obtain a mixture of iodoamino acids and iodopeptides (and iodide) by partial hydrolysis.

At the end of the hydrolysis, carriers were added ($I^-$, MIT, DIT, T3, T4; 5 $\mu$g each). The hydrolyzate was clarified by centrifugation at 1200 × *g* for 10 min at 4° and the supernatant was used directly for chromatography.

*Gel chromatography*

Sephadex gels (Pharmacia, Uppsala, Sweden) were used. Sephadex G-10 (batch Nos. 423, 1963, 3411 and 5560) and Sephadex G-25M (batch Nos. 259 and 390) were allowed to swell in an excess of distilled water with constant stirring at 19–20° for at least 3 h. During the swelling period, the fines were discarded by three successive decantations. Glass columns with circulating jackets were used to obtain either 20 × 2.4 cm or 40 × 1 cm gel beds. The swollen gel was poured into the column and equilibrated for at least 2 h with at least two void volumes of the appropriate eluent and at the temperature selected for elution.

In some experiments, the void volume ($V_0$) of the column was determined before chromatography by measuring the elution volume of a 0.2% solution of Blue Dextran 2000 (Pharmacia). The blue dextran solution was always freshly prepared in the eluent.

Sample sizes were between 0.5 and 3 ml and elution was carried out at a constant flow-rate of either 0.33 or sometimes 0.9 ml/min. This flow-rate was obtained with a pump (Büchler micro-pump 2.6000) except for eluent 2, which was fed to the column with a Mariotte flask. Fractions of 2 ml were automatically collected with a Seive fraction collector.

Biological samples (thyroid dialyzates or hydrolyzates), labelled *in vivo* with $^{125}I$, were supplemented with trace amounts of [$^{131}I$]iodide before chromatography in order to control both the $K_{av}$ value and the chromatographic recovery of iodide. Radioactive standards were generally chromatographed in pairs, one member being labelled with $^{131}I$ and the other with $^{125}I$.

*Radioactivity countings*

The radioactivity content of each collected fraction ($^{131}I$ and/or $^{125}I$) was measured with a Packard (Downers Grove, Ill., U.S.A.) Autogamma spectrometer with a yield of 35% for $^{131}I$ and 37% for $^{125}I$.

*Expression of the results*

Results were obtained in the form of chromatograms consisting of peaks of

radioactivity. Because of the difference in the counting yields for $^{131}I$ and $^{125}I$, and the different amounts of radioactivity used throughout this work, each chromatogram was normalized as follows. For radioactive standards, the radioactivity counts were represented on a modified scale so that all the peaks were given the same height. Then, for each peak, each sample was drawn as a percentage of the maximum value. For biological materials, each sample was generally drawn as a percentage of the total radioactivity eluted.

The recovery of each radioactive standard was calculated by taking its deiodination into account and was expressed as a percentage of the true radioactivity layered on the top of the column. For each iodo-compound also, the elution volume ($V_e$) was obtained in millilitres and its $K_{av}$ value calculated.

## RESULTS

### *Choice of an eluting system*

This choice was made for a fixed temperature of 20°.

*Successive elutions with eluents 1 and 2.* This eluting system, as used for filtration on Sephadex G-25M, gives good separation of iodide, MIT and DIT[10,15] but does not resolve mixtures that also contain iodopeptides (Fig. 1). From this result, our first assay was simply to use it with Sephadex G-10. Table I compares the results obtained with Sephadex G-25M and G-10.

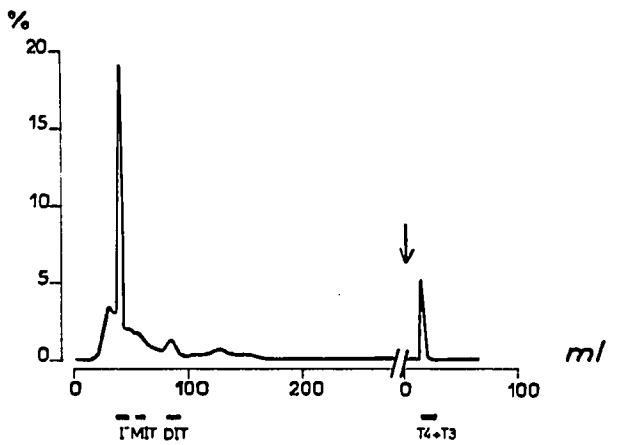


Fig. 1. Chromatography at 20° on Sephadex G-25M of a rat thyroid dialyzate labelled with $^{125}I$ and prepared as described in the text. Sample size: 1 ml. Flow-rate: 0.33 ml/min. The column (40 × 1 cm) was eluted successively with 0.2 *M* ammonium acetate buffer of pH 5.8 (eluent 1) and with *tert.*-amyl alcohol saturated with 2 *N* ammonia solution (eluent 2) as indicated by the arrow. Elution of the radioactive standards is indicated under the chromatogram by bars. Iodopeptides are poorly separated from other iodo-compounds.

In the elution of iodide, MIT and DIT with eluent 1, the chromatographic recoveries of MIT and DIT are similar for the two types of gels but the $K_{av}$ values are greater for Sephadex G-10 than for Sephadex G-25M. Also for iodide, the $K_{av}$ value is the greatest for Sephadex G-10, a situation which is favourable for a better

TABLE I

COMPARATIVE CHROMATOGRAPHY OF IODO-COMPOUNDS ON SEPHADEX G-25M AND G-10

Radioactive standards were separately chromatographed at 20° on a 20 × 2.4 cm column of either Sephadex G-25M or G-10 with 0.2 *M* ammonium acetate of pH 5.8 as eluent (eluent 1). Individual recoveries are given when only two determinations were made. Average values with their standard deviation are given when at least three determinations (number in parentheses) were made.

| *Iodo-compound* | *Sephadex G-25M* | | *Sephadex G-10* | |
|---|---|---|---|---|
| | $K_{av}$ | *Recovery (%)* | $K_{av}$ | *Recovery (%)* |
| Iodide | 1.21 ± 0.09 (7) | 100.1 ± 3.5 (4) | 2.99 ± 0.11 (5) | 60.8–60.6 |
| MIT | 1.98 ± 0.15 (5) | 97.0–101.9 | 3.62 ± 0.31 (3) | 97.8–100.0 |
| DIT | 3.20 ± 0.13 (5) | 98.1–98.9 | 8.02 ± 0.20 (3) | 98.1–101.2 |

separation from iodopeptides. Nevertheless, its recovery is lower than for Sephadex G-25M.

In the elution of hormones with eluent 2, the hormones (T3 and T4) are partially separated with Sephadex G-10 whereas they are eluted together when Sephadex G-25M is used (Fig. 2). On the other hand, the recoveries are only about 10% and 24% for T4 and T3, respectively, with Sephadex G-10, whereas they are almost 100% with Sephadex G-25M.

This eluting system (eluents 1 and 2), which is routinely used with Sephadex G-25M, is not as suitable with Sephadex G-10.

*Successive elutions with eluents 3 and 4.* This eluting system is widely used to separate iodide and iodinated amino acids by partition chromatography on paper[8] (Partridge's system). By combining partition chromatography with the molecular sieving of Sephadex G-10, it was hoped to separate also iodopeptides from other iodo-compounds.

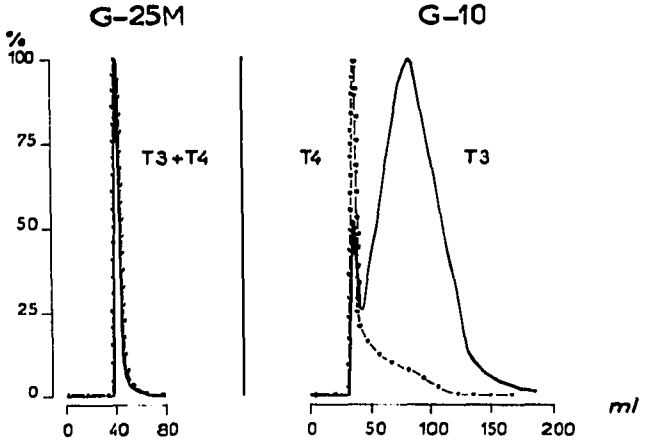


Fig. 2. Comparative chromatography at 20° of T3 and T4 on either Sephadex G-25M or Sephadex G-10 with *tert.*-amyl alcohol saturated with 2 *N* ammonia solution (eluent 2). Samples size: 3 ml. Flow-rate: 0.9 ml/min. Column size: 20 × 2.4 cm. With Sephadex G-10, a slight separation is obtained.

In the elution of iodide, MIT and DIT with eluent 3, a preliminary experiment made with a first batch of Sephadex G-10 showed that the successive elution of iodopeptides, iodide, MIT and DIT occurred with a recovery of 100% except for iodide (72.8 ± 0.5%). Nevertheless, with other batches of Sephadex G-10 a good separation between iodide and MIT was no longer obtained. Addition to solvent 3 (used for equilibration and thereafter for elution of the column) of carrier iodide and sodium chloride at concentrations of 10 μg/ml and 0.15 *M*, respectively, again enabled a good separation to be obtained (eluent 5). The carrier iodide both diminishes the tailing and improves the recovery of the radioactive iodide. Moreover, at higher concentrations, it also improves the separation between iodide and MIT even in the absence

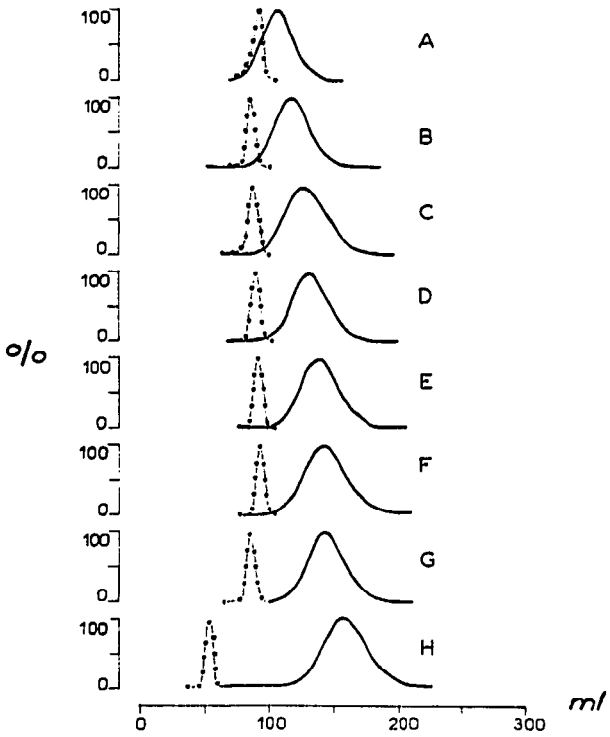


Fig. 3. Chromatography of iodide labelled with $^{131}I$ (●—●) and MIT labelled with $^{125}I$ (———) on Sephadex G-10 at 4°. Column size: 40 × 1 cm. Sample size: 1 ml. Flow-rate: 0.33 ml/min. Different elution patterns are obtained with eluent 3 when modified as follows: A, no modification; B, addition of sodium chloride (0.01 *M*); C, addition of sodium chloride (0.05 *M*); D, addition of sodium chloride (0.15 *M*); E, addition of sodium chloride (0.15 *M*) and potassium iodide (10 μg/ml of iodide); F, addition of sodium chloride (0.15 *M*) and potassium iodide (50 μg/ml of iodide); G, addition of sodium chloride (0.15 *M*) and potassium iodide (500 μg/ml of iodide); H, addition of potassium iodide (500 μg/ml of iodide).

of sodium chloride. Fig. 3 shows chromatograms obtained with different mixtures of salts added to solvent 3. A solvent containing potassium iodide (10 μg/ml of iodide) and sodium chloride (0.15 *M*) will give the best analysis of a biological sample containing iodopeptides, iodide, MIT and DIT. However, a solvent containing no sodium

chloride but with carrier iodide at a concentration of 500 $\mu$g/ml of iodide will be more suitable for samples of great volume and containing no iodopeptides.

In the elution of hormones with eluent 4, both hormones (T3 and T4) are eluted together with a recovery of 100%.

In the elution of iodopeptides, the study was carried out on rat thyroid dialyzates, which are known to be very rich in iodopeptides but very poor in MIT and DIT. Figs. 1 and 4 give a comparison of the results obtained with Sephadex G-25M and

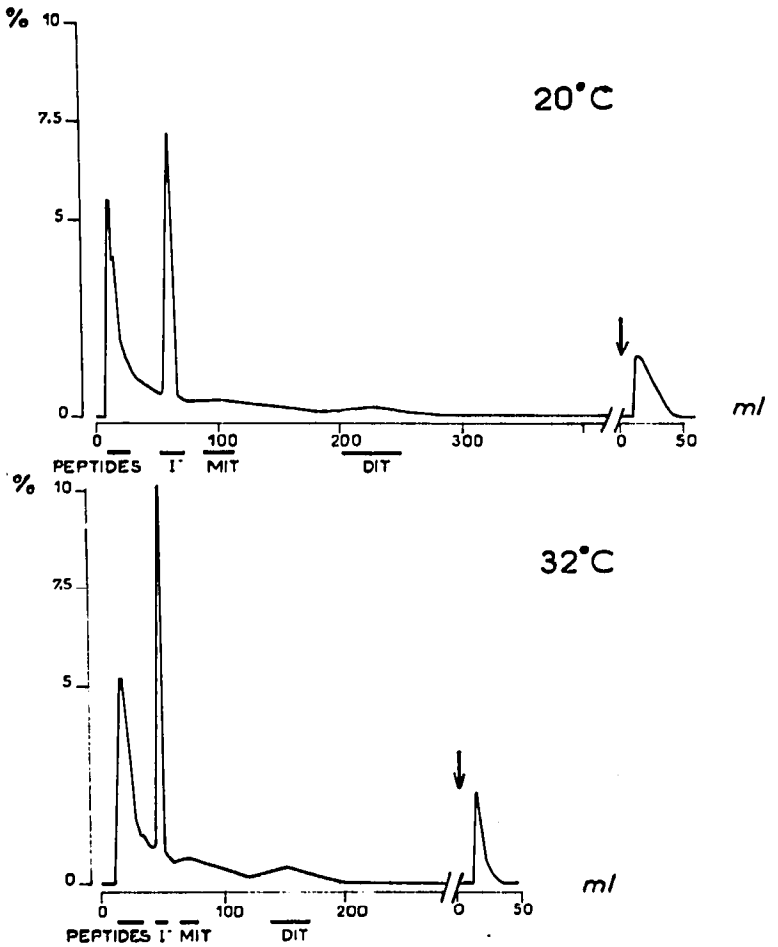


Fig. 4. Chromatography of rat thyroid dialyzates on Sephadex G-10 at 20° and 32°. Column size: 40 × 1 cm. Sample size: 1 ml. Flow-rate: 0.33 ml/min. Elution was carried out with eluents 5 and 4 (arrow) successively. Dialyzates were labelled *in vivo* with $^{125}$I and prepared as described in the text.

G-10, respectively. With Sephadex G-25M, the peaks of iodopeptides overlap with those of iodide (the peak of which is considerably higher than with Sephadex G-10) and of iodotyrosines, making it impossible to measure MIT accurately. Some iodopeptides even have a $K_{av}$ value greater than that of DIT. With Sephadex G-10, the

separation of iodopeptides and iodide is good and no iodopeptides are eluted after DIT, but MIT is probably slightly contaminated with a few iodopeptides which are not excluded by the gel.

*Influence of temperature*

The temperature effects were tested at 4°, 20° and 32°.

*Elution of iodopeptides.* Fig. 4 shows chromatograms obtained with thyroid dialyzates at 20° and 32°. After correction for the incomplete recovery of iodide and taking into account the 100% recovery of MIT and DIT, it was calculated that the recovery of iodopeptides was 100% for the three temperatures tested.

As can be seen by comparing Figs. 4 and 1, increasing the temperature to 32° for Sephadex G-10 gives a similar overlapping chromatogram to that for Sephadex G-25M at 20°. From this result, it appears that a lower temperature would be a better condition for the separation of iodopeptides. As seen later, a temperature of 4° has been tested for thyroid hydrolyzates.

*Elution of iodide, MIT and DIT.* As shown in Fig. 5, the $K_{av}$ values decrease

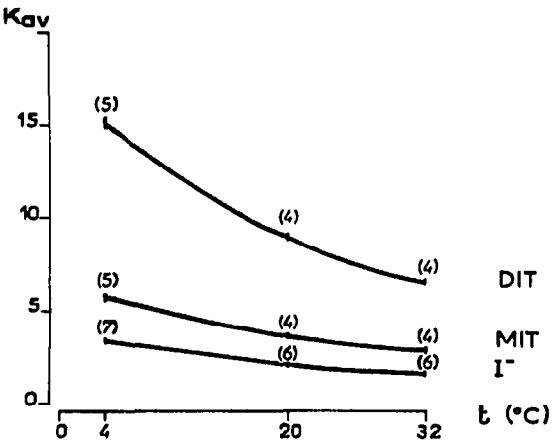


Fig. 5. Chromatography of radioactive standards iodide, MIT and DIT on Sephadex G-10 with eluent 5 at 4°, 20° and 32°. Column size: 40 × 1 cm. Sample size: 1 ml. Flow-rate: 0.33 ml/min. All $K_{av}$ values (the number of measurements is given in parentheses) decreased as the temperature increased.

when the temperature is increased from 4° to 32°. Simultaneously, the width of the peaks also decreases and consequently the separation remains good in spite of the decreased $K_{av}$ values. The chromatographic recoveries are all 100%, except for iodide, for which the recovery decreases from 89.6 ± 0.4% at 4° to 66.8 ± 2.8% at 32°.

*Elution of hormones.* Both hormones (T3 and T4) are eluted in a single asymmetrical peak, the width of which decreases when the temperature is increased. The recovery for each hormone is 100% at all temperatures.

*Application to thyroid hydrolyzates*

Three rat thyroid hydrolyzates were chromatographed at 4°, two hydrolyzates at 20° and two others at 32°. Fig. 6 shows an example for each temperature.

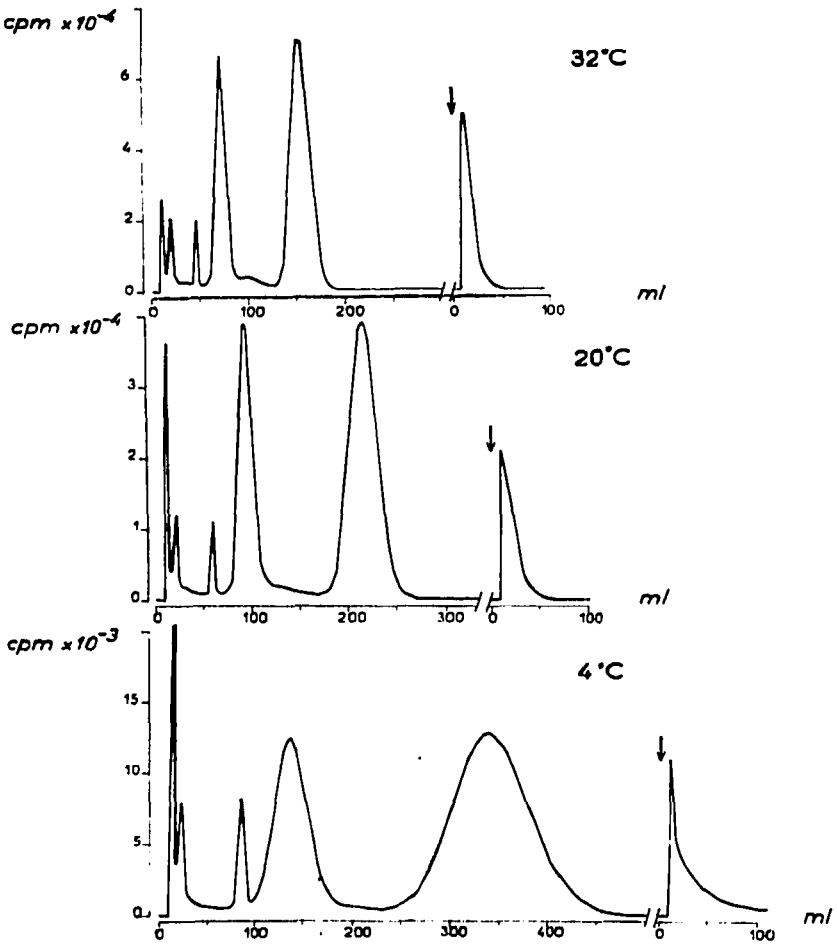


Fig. 6. Chromatography of rat thyroid hydrolyzates on Sephadex G-10 at 4°, 20° and 32°. Column size: 40 × 1 cm. Sample size: 0.5 ml. Flow-rate: 0.33 ml/min. Elution was carried out with eluents 5 and 4 (arrow) successively. Hydrolyzates were labelled *in vivo* with $^{125}I$ and prepared as described in the text.

By contrast to dialyzates, iodopeptides resolved into two main peaks. The first peak was eluted with the $V_0$ and the second was eluted at about 1.5 $V_0$. By calculating the ratio of the heights of peaks 2 and 1, it was observed that the relative importance of peak 2 increases with increasing temperature (0.27 at 4°, 0.30 at 20° and 0.70 at 32°). Simultaneously, a new small peak appears between MIT and DIT at 20° and 32°. It is concluded that for iodopeptides the most suitable of the three temperatures of chromatography tested is 4°. As for iodotyrosines and hormones, their separation is good at all three temperatures tested but becomes more rapid as the temperature increases.

The recovery of iodide is dependent on temperature but also on the nature of the sample being analyzed. As shown in Fig. 7, the recovery of iodide decreases more

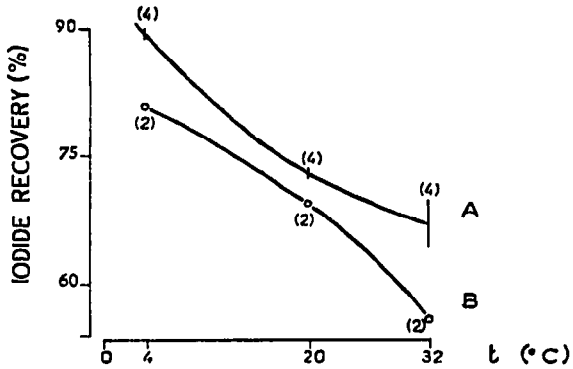


Fig. 7. Variation with temperature of the recovery of radioactive iodide after chromatography on Sephadex G-10 with eluent 5 and for two conditions of the medium: A, radioiodide dissolved in 1 ml of 0.09 *M* phosphate buffer of pH 7.2; B, radioiodide added to 1 ml of rat thyroid hydrolyzate in solution in 0.09 *M* phosphate buffer of pH 7.7. The iodide recovery was always lower in the presence than in the absence of thyroid hydrolyzate for the three temperatures tested. The number of measurements is given in parentheses.

rapidly with temperature when total hydrolyzate is present than when phosphate buffer is present alone in the sample.

Table II gives the results obtained after correction for the recovery of iodide from the seven thyroid hydrolyzates. As tested by covariance analysis between temperature groups, the distribution of radioactivity between iodo-compounds is independent of temperature in spite of the difference in the duration of *in vivo* labelling (24 or 48 h).

Expressed as a percentage of the total radioactivity, the total recovery of hy-

TABLE II

CHROMATOGRAPHY OF RAT THYROID HYDROLYZATES ON SEPHADEX G-10

Radioactive rat thyroid hydrolyzates prepared as described in the text were chromatographed on 40 × 1 cm columns of Sephadex G-10 with an aqueous phase of Partridge's solvent (*n*-butanol–water–acetic acid, 78:17:5) supplemented with carrier iodide (10 μg/ml) and sodium chloride (0.15 *M*) (eluent 5). Of the 93% of total radioactivity recovered, about 18% was eluted in the second eluent and is not indicated in the table.

| *Temperature (°C)* | *Recovery (%)* | | | | | |
|---|---|---|---|---|---|---|
| | *Total* | *Bound to the gel* | *Iodopeptides* | *$I^-$* | *MIT* | *DIT* |
| 32 | 93.78 | 6.22 | 5.53 | 4.02 | 23.24 | 45.36 |
| | 93.56 | 6.44 | 5.84 | 4.01 | 23.63 | 43.19 |
| 20 | 96.60 | 3.40 | 8.00 | 3.44 | 25.77 | 41.29 |
| | 90.30 | 9.70 | 5.38 | 2.57 | 22.10 | 41.38 |
| 4 | 94.00 | 6.00 | 5.50 | 3.31 | 20.09 | 44.99 |
| | 93.35 | 6.65 | 5.16 | 2.76 | 21.13 | 42.43 |
| | 92.00 | 8.00 | 4.99 | 3.95 | 19.22 | 45.16 |
| Average ±S.D. | 93.37 ± 1.93 | 6.63 ± 1.93 | 5.77 ± 1.02 | 3.43 ± 0.60 | 22.16 ± 2.25 | 43.40 ± 1.78 |

drolyzates averaged 93%. About 7% of the total radioactivity was not eluted with the two eluents. This strong binding is independent of the temperature.

The results of the elution of hormones are not given in Table II because eluent 4 was used only once at 32°. In this instance, the radioactivity eluted was about 18%. In all other instances, another eluent which allows complete separation of T3 and T4 was used, and the results will be published in a later paper.

## DISCUSSION

Various techniques have been already described for the separation of mixtures of iodinated amino acids. By combining partition chromatography and molecular sieving on Sephadex G-10, we have developed a method which permits the analysis of samples containing iodopeptides, iodide, MIT, DIT and iodothyronines on a single column.

### *Pre-treatment of the sample*

Before gas chromatography, iodinated tyrosines and thyronines must be converted into volatile derivatives after silylation[1–4].

As discussed by Faircloth *et al.*[7], before TLC the thyroid digest is first concentrated by passing it through a Dowex 50 cation-exchange column; the contents are eluted and then redissolved in a small volume of 2 *N* ammonia solution. In Sephadex LH-20 column separation, the same procedure was used by Williams *et al.*[5] as a preliminary purification step for removing lipids and inorganic iodide from the enzymatic digest (and probably iodopeptides). Such tedious pre-treatments may lead to loss of iodo-compounds and do not allow the direct determination of iodide content.

In our method, such disadvantages do not occur because direct layering of the sample is possible and no removal of lipids and iodide is necessary.

### *Sample volume*

Paper chromatography and TLC require samples of small volumes.

We have routinely applied samples of 0.5–1.0 ml to 40 × 1 cm columns of Sephadex G-10. Preliminary experiments (to be published elsewhere) have shown that iodide, MIT and DIT were separated from samples as large as 50 ml on 40 × 1 cm columns and with an eluent containing carrier iodide at a concentration of 500 μg/ml of iodide.

### *Reproducibility and duration of chromatography*

The reproducibility is generally good with Sephadex filtrations. In our work, the reproducibility was very good and under each set of conditions tested the relative standard deviation of $K_{av}$ was never higher than 2%. The recovery was always 100% for each iodinated molecule except for iodide. The recovery of iodide is dependent on temperature but is satisfactorily reproducible as the relative standard deviation of the recovery was never higher than 4%.

Depending on the technique used, the duration of chromatography is very different. With two-dimensional TLC, the total development of the plate requires about 4.5 h[7], and to this duration must be added the time required for air drying after

each solvent and for pre-treatment of the sample on a Dowex column and by lyophylization. The same pre-treatment is applied to the sample before separation on a Sephadex LH-20 column, at least 14 h being required for column chromatography[5].

In our work, a good separation with symmetrical peaks was obtained only when using relatively low flow-rates of the order of 0.33 ml/min. Consequently, a complete analysis from the iodopeptides to the hormones will be obtained in 16 h at 32° and 32 h at 4°. Nevertheless, this relatively long duration is counterbalanced by the facts that a total analysis on a single column is achieved and pre-treatment of the hydrolyzate is avoided.

*Loss of material from thyroid hydrolyzates*

With the TLC technique, the recovery of compounds applied to the plate averaged 76%[7] and 80%[9]. With the Sephadex LH-20 technique, the recovery varied from 86.9 to 98.7%[5] of the iodine-containing compounds remaining after Dowex pre-treatment and applied to the Sephadex column.

In our work, the recovery was consistently about 93% whatever the temperature of chromatography. Hence 7% of the total radioactivity layered on the top of the column was firmly bound to the Sephadex gel. The molecular form of this bound fraction is unknown.

*Separation of iodopeptides*

In paper chromatography[8] and TLC[9], the presence of unknown compounds may be detected in rat thyroid hydrolyzates. They migrate with or between MIT and DIT and are probably iodopeptides.

In this work, rat thyroid dialyzates were used as reference media containing large amounts of iodopeptides. In comparison with Sephadex G-25M, the analysis of a thyroid dialyzate using Sephadex G-10 gives smaller amounts of MIT and DIT although their recovery is 100% (Fig. 4). Contamination of these iodotyrosines (and also of iodide) by iodopeptides is much less with Sephadex G-10 than with Sephadex G-25M. It is therefore concluded that, although not perfect, the method using Sephadex G-10 as described here will give the best results with a sample containing a large amount of iodopeptides.

Iodopeptides of thyroid hydrolyzates are separated into two main peaks (Fig. 6). The first peak, eluted in the volume $V_0$, is composed of excluded iodopeptides. The second peak is composed of iodopeptides which are reversibly bound to the gel either because their molecular weights are less than the exclusion molecular weight of the gel (about 700) or because they contain aromatic amino acids such as tryptophan, tyrosine and histidine[16–19] or iodotyrosines and iodothyronines. On increasing the temperature of chromatography, it was observed that the second peak of iodopeptides increased and that a new small peak appeared between MIT and DIT. It is possible that at increased temperatures, the pore size becomes larger and so permits permeation by larger iodopeptides, or that the binding of these iodopeptides is an exothermic process.

*Separation of iodinated amino acids*

Brook and Housley[20] demonstrated that halogen-substituted phenols are strongly adsorbed on Sephadex G-10. Our results show that DIT is more adsorbed than MIT

and consequently the separation of these two iodotyrosines occurs. Furthermore, both iodothyronines are more strongly adsorbed than iodotyrosines on the gel, a situation which is similar to that encountered with Sephadex G-25M.

*Versatility*

Gas chromatography has been used only for the separation of radioactive standards[1-4]. All other methods are applicable to thyroid hydrolyzates.

Although our method was devoted mainly to the analysis of thyroid hydrolyzates, it can also be used for the analysis of other biological samples with some modifications. The temperature of chromatography may be selected according to the assumed composition of the sample to be analyzed. In particular, if the sample is rich in iodopeptides, chromatography at 4° will be the most appropriate. In contrast, for a sample that contains no iodopeptide, chromatography at 32° will give a more rapid separation of iodide and iodotyrosines. It was observed that a trichloroacetic acid supernatant prepared from a rat thyroid homogenate may be chromatographed after its pH is increased to 3. Butanol-extractable iodine was also analyzed by this technique on rat plasma. Iodide, T3 and T4 were separated without previous concentration of the sample. Also, MIT and DIT would be separated if they were present. Finally, it is noteworthy that total thyroid homogenate would be analyzed directly.

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